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The fate of isolated blastomeres with respect to germ cell formation in the amphipod crustacean *Parhyale hawaiensis*

Cassandra G. Extavour*

Department of Zoology, Laboratory for Development and Evolution, University of Cambridge, Downing Street, Cambridge, CB2 3EJ Cambridgeshire, United Kingdom

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Abstract

Germ cells may be specified through the localization of germ line determinants to specific cells in early embryogenesis, or by inductive signals from neighboring cells to germ cell precursors in later embryogenesis. Such determinants can be produced and localized during or after oogenesis, either autonomously by oocytes or by associated nutritive cells. In *Drosophila*, each oocyte is connected to nurse cells by cytoplasmic bridges, and determinants synthesized in nurse cells are transported through these bridges to the oocyte. However, the *Drosophila* model may not be applicable to all arthropods, since in many species of all four extant arthropod classes, gametogenesis functions without nurse cells. In this paper, I use immunodetection of Vasa protein to study germ cell development in the amphipod crustacean *Parhyale hawaiensis*, a species whose ovaries lack nurse cells and whose eggs lack obvious polarity. Previous cell lineage analyses have shown that all three germ layers and the germ line are exclusively specified by third cleavage. In the present study, I use a molecular marker to follow germ cell development during *P. hawaiensis* embryogenesis. I determine the capacity of individual blastomeres to form germ cells by isolating blastomeres at early cleavage stages and provide experimental evidence for localized germ cell determinants at the two-cell stage in *P. hawaiensis*. These experiments indicate that many aspects of early amphipod development, including timing and symmetry of cell division, the transition from holoblastic to superficial cleavage, and possibly some gastrulation movements, are cell autonomous following first cleavage.

Keywords: Germ cells; Crustacean; *Parhyale hawaiensis*; Preformation; Experimental embryology

Introduction

The correct specification of germ cells is a developmental sine qua non for the reproduction and evolution of all sexually reproducing animals. It is therefore critical to understand not only how germ cells are specified in model organisms, but also the extent of variation in the mechanisms specifying germ cells in diverse metazoans. Is germ line establishment so "constrained" that there is very little variation in its developmental program, or are there as many ways of guaranteeing gamete production as there are embryonic cleavage programs, ovarian morphologies, or metazoan phyla?

In some animals, germ cell specification is achieved by inductive signaling during embryogenesis: undifferentiated cells receive an inductive signal from neighboring tissues and respond by differentiating as germ cells. This epigenetic type of specification has been demonstrated experimentally only in mice and axolotls (Lawson et al., 1999; Nieuwkoop, 1947; Tam and Zhou, 1996; Ying and Zhao, 2001; Ying et al., 2000), but a large body of descriptive evidence suggests that it may be a widespread and possibly ancestral mechanism of metazoan germ cell specification (Extavour and Akam, 2003). In contrast, many animals specify their germ line very early in embryogenesis by the creation of a unique cytoplasm containing germ cell determinants in one region of the embryo (preformation). This "germ plasm" is inherited by

* Fax: +44 122336679.
E-mail address: cgme2@hermes.cam.ac.uk.

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only one or a few cells, primordial germ cells (PGCs), which become the unique founder population for the germ line (Extavour and Akam, 2003).

Many genetic model organisms specify germ cells by preformation, but while the germ line determinants contained in germ plasm appear to be highly conserved (Carré et al., 2002; Houston and King, 2000; Ikenishi, 1998; Knaut et al., 2000; Lehmann and Rongo, 1993; Tsunekawa et al., 2000), the mechanisms that ensure that germ plasm is manufactured and localized may be quite different. In *Drosophila melanogaster*, each oocyte is connected to nurse cells by cytoplasmic bridges, and germ plasm components synthesized in nurse cells are transported through these bridges to the oocyte (Rongo et al., 1997). In *Xenopus laevis*, cyst cells connected to the developing oocyte may similarly be responsible for ensuring oocyte polarity and germ plasm localization during oogenesis (Heasman et al., 1984; Kloc et al., 2004). Zebrafish oocytes do not have nurse cells, but the oocyte itself generates germ plasm, which is asymmetrically localized by the end of oogenesis (Knaut et al., 2000). Cytoplasmic components of germ plasm in *Caenorhabditis elegans* are likewise synthesized during oogenesis by the developing oocyte but do not become asymmetrically localized until after fertilization (Strome and Wood, 1983). Thus, while establishment of polarity, including germ plasm localization, is clearly not dependent on nurse cells, we currently have no functional information on how germ cell specification is achieved in nurse cell-free arthropod systems. The nurse cell-driven model provided by *D. melanogaster* is unlikely to be representative of most arthropods or even most insects, given the wide variation in arthropod ovarian morphology, modes of gametogenesis, and early embryogenesis.

Parhyale hawaiensis is an amphipod crustacean of the class Malacostraca. Its ease of laboratory culture, embryonic and molecular manipulation, well-defined cell lineage during development (Gerberding et al., 2002), and promise for functional genetic analysis (N. Patel, M. Averof, A. Pavlopoulos, personal communication) make it an excellent model in which to study comparative aspects of crustacean and arthropod development. Amphipod early cleavage patterns are stereotyped, radial, and holoblastic. Cell lineages defining all three germ layers are predictable as early as the eight-cell stage in *P. hawaiensis* (Gerberding et al., 2002) and in other amphipods of the same family (Wolff and Scholtz, 2002). However, it is not known how cell lineage is coupled to cell fate for any of these eight cells. For example, lineage analysis has suggested that the germ line derives from a single cell at this eight-cell stage (Gerberding et al., 2002; Wolff and Scholtz, 2002), but how this cell acquires germ cell fate is unknown. Amphipod ovaries lack nurse cells, and it is unclear when and how zygotic asymmetries are generated, whether autonomously during oogenesis or by cytoplasmic rearrangement after fertilization.

Germ cells can often be traced through embryogenesis from initial specification to gonad colonization using the mRNA or protein encoded by the *vasa* gene as a molecular marker. *Vasa* was first identified in *D. melanogaster* as a gene encoding an ATP-dependent DEAD box helicase that is necessary, but not sufficient, for germ cell formation, survival, and some aspects of oogenesis (Hay et al., 1988; Lasko and Ashburner, 1988; Styhler et al., 1998). *Vasa* expression is generally specific to germ cells throughout both embryonic development and adult reproductive life in almost all metazoans in which homologues have been identified (Extavour and Akam, 2003). There are only two known exceptions to the general rule that *vasa* gene products are found specifically in germ cells: (1) in some insects, *vasa* products are detected briefly in all regions of the early embryo, but become restricted to germ cells (Chang et al., 2002; Hay et al., 1988; Lasko and Ashburner, 1990; Nakao, 1999); (2) in animals that have adult somatic stem cells capable of generating several different cell types, including gametes, *vasa* family genes are sometimes expressed in such pluripotent cells (Mochizuki et al., 2001; Shibata et al., 1999). Unlike generic stem cell genes such as the *piwi* family (Lin and Spradling, 1997), or genes which may have both germ cell and somatic functions such as the *nanos* genes (Curtis et al., 1995; Lehmann and Nusslein-Volhard, 1991; Pilon and Weisblat, 1997; Wang and Lehmann, 1991), the germ cell-specific expression and function of *vasa* family members make them a good tool to identify germ cells in organisms with unknown germ line origin.

In this study, I use Vasa protein as a marker to identify primordial germ cells in *P. hawaiensis* from their specification at the eight-cell stage until the end of embryogenesis. To determine the mechanism of germ cell specification in early embryos, I use classical experimental embryological techniques to establish the developmental potential of individual blastomeres at the two-, four-, and eight-cell stages, with respect to germ cell formation. I show that only one cell at the two-cell stage, the cell that will give rise to the germ line founder cell at the eight-cell stage, contains a cytoplasmic germ line determinant. I also demonstrate that acquisition of germ cell fate is autonomous and unique to this germ cell precursor, and that other blastomeres cannot give rise to germ cells even if the germ cell precursor is ablated. In contrast to what has been observed for many marine invertebrates, including a crustacean, after first cleavage development of this crustacean is not regulative with respect to germ cell fate, and distinct cell fates have been autonomously assigned to each cell at the two-cell stage.

Materials and methods

Animal husbandry

Populations of *P. hawaiensis* were raised from a seed colony kindly supplied by Ernst Wimmer (originally from

Nipam Patel's lab). Animals were kept at 25°C in large plastic tubs with a bed of crushed coral (CaribSea) in artificial seawater (ASW). ASW was 30 g Tropic Marin salts/L deionized water, to a final salinity of 1.022–1.024. Water was changed every 2 weeks, and animals were fed ground fish flakes (TetraFin), freeze-dried Tubifex (Inter-Pet), and algae pellets (PlecoChips) every few days.

Developmental staging

Mating pairs of males and females were maintained separately until fertilization and egg laying occurred. Females carrying eggs were anaesthetized for a few minutes with clove oil diluted 1:1000 in filtered ASW (FASW). Embryos were prised out of the ventral brood pouch with forceps, placed in 3 cm plastic petri dishes in FASW, kept at 18 or 25°C, and monitored several times daily until hatching, with one water change at least every 3 days.

Immunohistochemistry

Embryos removed from the brood pouches of females were either dissected and fixed immediately or cultured (as above) until the desired developmental stage was reached. Embryos were immersed in a drop of fixative [4% solid paraformaldehyde (PolySciences EM grade) in FASW with a salinity of 1.026–1.028 (PFASW) or 3.7% formaldehyde diluted from 37% liquid stock (Sigma) in FASW of the same salinity as the cultures (1.022–1.024)] on a silicone rubber plate (Sylgard® 184, Dow Corning). Membranes and embryonic cuticles were removed with electrolytically sharpened tungsten needles. After fixation embryos were either processed immediately for immunohistochemistry or dehydrated through a methanol series and stored at −20°C.

Antibody staining was performed using standard protocols (Patel, 1994). Primary antibodies used were rabbit For2 (anti-Vasa) (Chang et al., 2002) 1:30; mouse D1α (anti-α-tubulin) (Sigma) 1:50–1:200; mouse anti-phospho-histone 3 (Cell Signalling) 1:500; and rabbit anti-phospho-histone 3 (Upstate Biotech) 1:200–1:400. Secondary antibodies used were all raised in goat: anti-rabbit Alexa 488; anti-rabbit Alexa 568; anti-mouse Alexa 488; anti-mouse Alexa 568 (Molecular Probes); anti-rabbit horseradish peroxidase; anti-rabbit alkaline phosphatase; anti-mouse Cy5, and anti-mouse Texas Red (Jackson ImmunoLabs). Counterstains used were TO-PRO-3 iodide 1 mM in DMSO, YO-PRO-1 iodide 1 mM in DMSO (Molecular Probes), and DAPI 0.5 μg/ml in deionized water (Sigma). When YO-PRO-1 iodide was used as a counterstain, 1 μg/ml RNaseA was added to the secondary cocktail. All fluorescent secondary antibodies were diluted 1:500, all enzymatically coupled secondary antibodies were diluted 1:300, and all counterstains were diluted 1:1000. Samples were mounted in Vectashield (fluorescent stains) or in 70% sterile glycerol in 1 × PBS (enzymatically developed stains).

Image capture and analysis

Dissected and stained embryos were viewed with a Zeiss Axioplan or Zeiss Axiophot upright compound microscope. Live embryos and blastomere isolates were viewed with a Leica MZFLIII stereomicroscope. Epifluorescent, bright field, incident light and DIC images were captured using a Nikon CoolSnap, Leica DFC300F, or Hamamatsu Orca monochrome digital camera, driven by OpenLab (Improvision) or Leica FireCam (Leica Microsystems). Samples for confocal microscopy were imaged using a Leica TCS confocal scanning microscope.

Two-dimensional projections, 3D reconstructions, orthogonal sections, nuclear counts, and QuickTime movies were generated using Volocity (Improvision). Figures were assembled using Photoshop 7.0.

Blastomere isolations

Early cleavage embryos were cultured (as above) until the desired cleavage stage. All dissections and cultures were carried out in FASW + 50 μg/ml ampicillin, in 3 or 5 cm plastic Petri dishes coated with 1% agarose in FASW. Membranes were removed using forceps and blastomeres separated from each other using an eyelash. Individual blastomeres and aggregates of blastomere derivatives were transferred between solutions using an agarose/FASW-coated mouth pipette. Isolated blastomeres were grown at 25°C for 24-36 h, then fixed for 15–30 min and stained as described above.

Results

P. hawaiensis embryonic development

Fig. 1A presents a brief description of embryonic development in *P. hawaiensis*. For a more detailed description of early development, the reader is referred to the studies of other researchers on this species (Browne, 2003; Gerberding et al., 2002) and other closely related marine amphipods (Langenbeck, 1898; Scholtz, 1990; Scholtz and Wolff, 2002; Weygoldt, 1958). Fig. 1A depicts the cycle of embryonic stages, which can be classified in terms of percentage of embryonic development completed or total number of hours from fertilization at a given temperature. Embryonic development of *P. hawaiensis* takes between 12 and 14 days at 25°C and slightly longer at 18°C (Fig. 1B). Initial cleavages are meridional and holoblastic. The first two cleavages are slightly unequal, such that one of the blastomeres at the four-cell stage is usually slightly smaller than the others. The third cleavage is very unequal, giving rise to four micromeres and four macromeres. In this paper, I will use the nomenclature suggested by (Gerberding et al., 2002), based on lineage derivatives, to identify the blastomeres up to third cleavage: the smallest macromere is

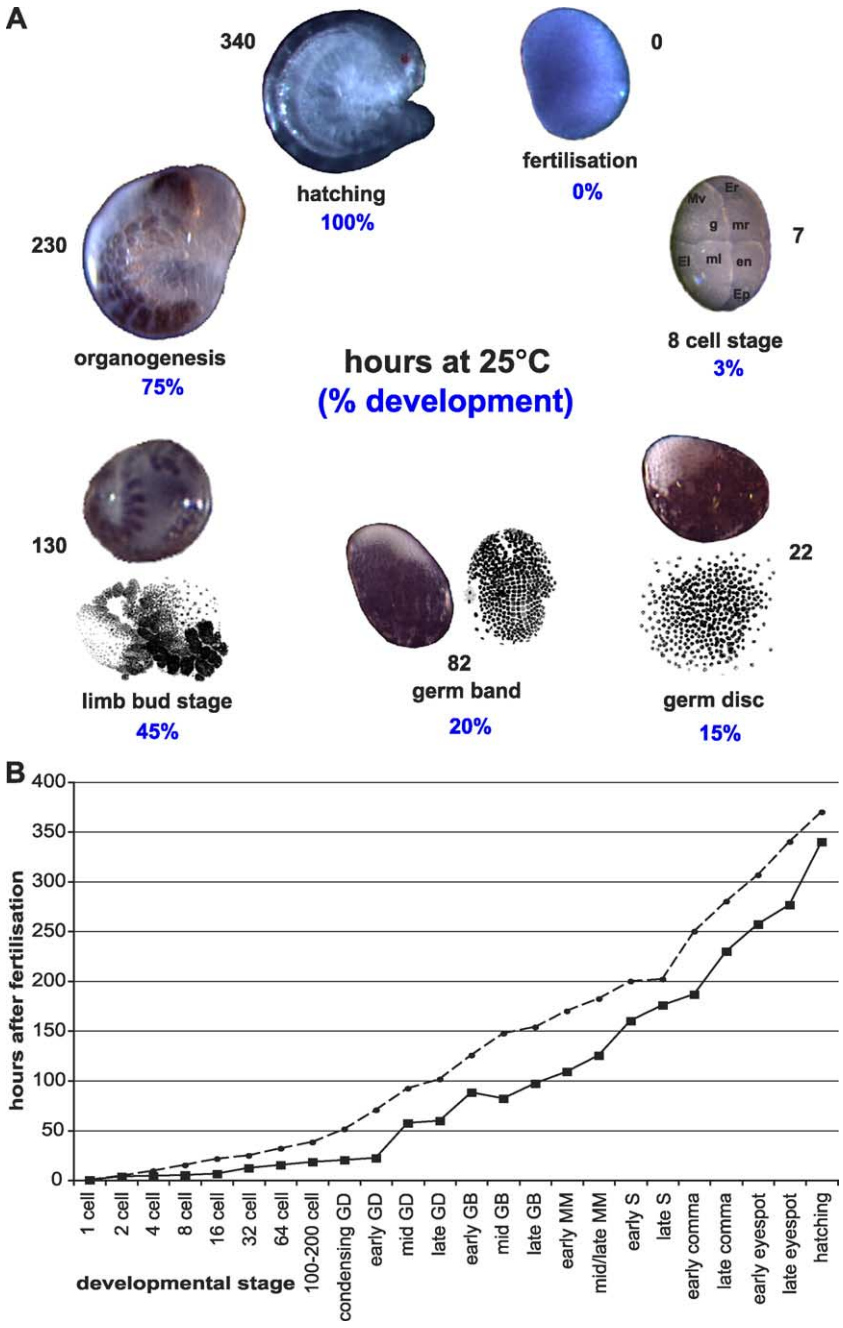


Fig. 1. Developmental staging in *P. hawaiensis*. (A) Live embryos (roughly to scale) at various stages from fertilization to hatching. Developmental stages are indicated as hours after fertilization at 25°C (black) and as percentage of total development (blue) (fraction of total time to hatching). For the germ disc, germ band, and limb bud stage, the distribution of nuclei of the embryonic rudiment/embryo is shown next to the live embryo. (B) Timing of embryonic development from fertilization to hatching. Data pooled from observations of embryos cultured at 18°C (dashed line; n = 349) or 25°C (solid line; n = 294).

called "Mv" (visceral mesoderm), the smallest micromere "g" (germ line), and the remaining macromere/micromere pairs are called "Er/mr" (right ectoderm/right mesoderm), "Ep/en" (posterior ectoderm/endoderm), and "El/ml" (left ectoderm/left mesoderm), labeling clockwise from "Mv" and "g." The second cleavage blastomeres will be referred to as "Mvg," "Emr," Epen," and "Eml."

When the embryo consists of approximately 100 cells, there is a transition from holoblastic to superficial cleavage, resulting in a monolayer of relatively yolk-free cells covering the surface of an anucleate globular mass of extruded yolk. There is a movement of cells over the egg surface to the anteroventral region, where most of the cells accumulate in a rough disc (15%), corresponding to the embryonic rudiment or germ disc. A few cells do not undergo this migration and instead are scattered over the remaining yolk surface, where they will contribute to extraembryonic structures. The germ disc proliferates until it consists of roughly 600–700 cells and then begins to elongate along the anteroposterior axis (20%), forming a

germ band similar to that seen in many insects and crustaceans (Johannsen and Butt, 1941; Weygoldt, 1994). Appendage development begins as outgrowths on the left and right sides of the germ band, while the anteroposterior elongation of the germ band curves the embryo around into an "S" shape such that the posterior of the embryo is close to the anterior head lobes (45%). As limb elongation continues, the embryo is curved into a "comma" shape with the ventral limbs in the curve of the "comma", and the embryo closes dorsally (75%). The hatchling emerges as a miniature version of the adult, with a fully formed gut and a complete set of appendages, and undergoes several moults until sexual maturity approximately 6 weeks after hatching.

A cross-reacting Vasa antibody identifies primordial germ cells (PGCs) throughout development

Outside of the insects, in animals without adult pluripotent somatic stem cells, *vasa* gene products are expected to be the best specific markers for primordial germ cells and developing gametes (see introduction). I used a cross-reacting antibody against Vasa family member proteins (Chang et al., 2002) to identify putative PGCs throughout *P. hawaiensis* embryonic development. The cells identified by this antibody correspond to those cells identified in previous cell lineage studies as being the exclusive descendants of the "g" micromere, which was proposed to be the germ line founder cell (Gerberding et al., 2002). The cell numbers, migratory behavior, and embryonic location at all stages of development of the Vasa-positive cells in this study correspond exactly to the lineage observations for "g" descendants (see descriptions below). I therefore conclude that this antibody specifically recognizes PGCs in *P. hawaiensis* and have used it to characterize germ cell development in this species.

PGCs during early P. hawaiensis embryonic development

The following section summarizes the main aspects of embryonic germ cell development. A more detailed analysis of germ cell proliferation and migration will be presented elsewhere. At the one-cell (Figs. 2A and B), two-cell (Figs. 2C and D), and four-cell stages (data not shown), no Vasa protein is detected in any region of the embryo. At the eight-cell stage, one of the micromeres expresses barely detectable levels of Vasa (Figs. 2E and F). This micromere can be identified as "g" on the basis of its smaller size relative to the other micromeres. Positive controls of embryos stained under the same conditions, but at later developmental stages, confirm that the absence of staining before the eight-cell stage is not an artifact (Figs. 2G and H).

When the germ disc begins to coalesce, four Vasa-positive cells (hereafter called PGCs) are located at the anterior edge of the germ disc (Fig. 3A). This position corresponds to the location of all "g" descendants at this stage, identified by cell lineage studies on *P. hawaiensis* (Gerberding et al., 2002). The PGCs are not in the same plane as the majority of the cells that comprise the germ disc but are instead slightly dorsal to the main disc (Fig. 3B; Supplementary Data Movie 1). As the cells of the germ disc divide, the PGCs also undergo mitosis and increase in number (Fig. 3C). At this stage, the PGCs are found clustered close together near the center of the germ disc (Fig. 3C). They lie between the ectoderm and the yolk, at the same level as mesendodermal cells (Fig. 3D; Supple-

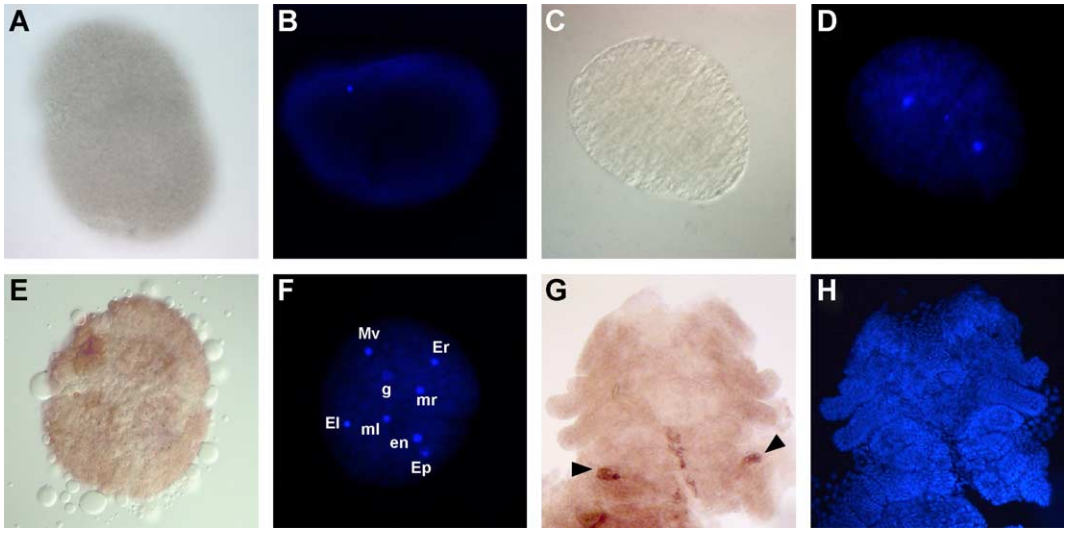
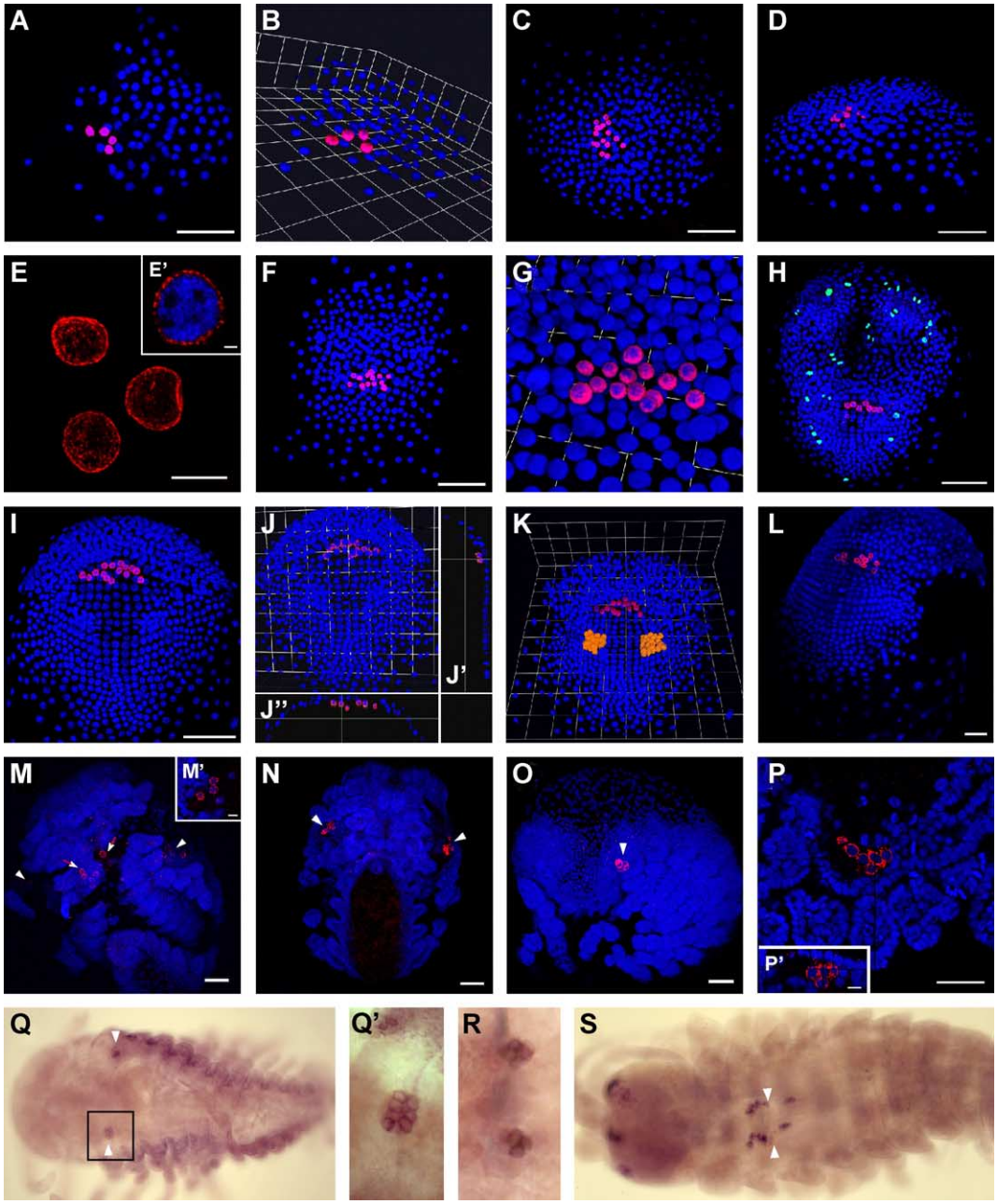


Fig. 2. Vasa protein expression in early cleavage stages. (A, C, E, and G) Bright field images of immunohistochemically developed embryos; (B, D, F, and H) corresponding DAPI stain showing nuclei (blue). (A and B) At the one- and two-cell (C and D) stages, no Vasa protein is detected. (E and F) At the eight-cell stage, Vasa protein is detected at low levels in the smallest micromere, "g". The "g" is identifiable not only by its small size (E), but also as its nucleus shows less compact chromatin with DAPI staining than the other blastomeres (F). (G and H) Positive control: Vasa-positive cells (arrowheads) are seen in later stage embryos stained under the same conditions as those shown in panels A–F.

mentary Data Movie 2). Vasa protein is concentrated in a punctate perinuclear pattern (Figs. 3E and E′). This distribution, characteristic of Vasa protein localization in several species (Batalova and Parfenov, 2003; Braat et al., 2000; Knaut et al., 2000), supports my interpretation that the cross-reacting Vasa antibody used specifically recognizes *P. hawaiensis* Vasa like proteins, and not other related DEAD box helicases.

When the germ disc begins its elongation and transformation to the germ band stage, PGCs remain clustered together but rearrange into an elongated row perpendicular to the anteroposterior axis of the embryonic rudiment (Fig. 3F). All PGCs are underneath the ectodermal cells of the germ disc. Anterior to the PGCs the embryonic rudiment is multilayered, with mesendodermal cells forming the inner layer; posterior to the PGCs the ectodermal cells are arranged in essentially a single layer with only a few underlying mesodermal precursors (Fig. 3G; Supplementary Data Movie 3). The germ band elongates and changes shape through cell rearrangement, mitosis, and possibly also localized apoptosis (C. Extavour, unpublished observations). The trunk region of the germ band elongates by mitosis of ectodermal cells, while the multilayered cells of the anterior region divide into two regions corresponding to the head lobes. By late germ band stages, the PGCs number 15 or 16 and remain between the future head and trunk regions underneath a single layer of ectodermal cells (Figs. 5H and I). Orthogonal sections of embryos at this stage



confirm that all PGCs are underneath the germ disc, and that the subcellular distribution of Vasa protein remains perinuclear (Figs. 3J, J′, and J″). Posterior to the PGCs, the germ band is mostly a single layer, with the exception of two groups of mesodermal precursor cells (mesoblasts) located approximately four to five ectodermal cell rows posterior to the PGCs (Fig. 3K).

PGCs during late P. hawaiensis embryonic development

Shortly before limb development begins, the PGCs separate into two clusters, left and right (Fig. 3L). These clusters are usually, but not always, roughly equal in cell number, with each cluster consisting of at least five PGCs. While limb development proceeds, the two clusters of germ cells migrate dorsolaterally towards the sides of the embryo, remaining surrounded by mesendoderm underneath the ectodermal cells (Figs. 3M and M′). By the time the tail end of the embryo has curled around towards the head region, all PGCs are located in one of two clusters, underneath the left and right lateral ectoderm (Fig. 3N). As the limbs elongate, the lateral clusters of PGCs continue migrating towards the dorsal side of the embryo (Figs. 3O and P), remaining close to each other in a cluster and covered by a single layer of lateral ectodermal cells (Fig. 3P′).

Just before eyespot development, when appendage development is almost complete, the PGC clusters come to lie on the dorsal side at the level of the first thoracic leg-bearing segment (Figs. 3Q and Q′). A few days before hatching when the eyespots are already visible, the clusters reach their final positions in two tight clusters on either side of the heart primordium at the dorsal midline (Fig. 3R). Prior to hatching, these clusters elongate posteriorly into two rows of cells that extend for at least two thoracic segments (Fig. 3S). The position of the PGCs at this stage corresponds to the final position of the descendents of "g" observed by lineage tracing (Gerberding et al., 2002) and to the position of the gonad rudiment in the hatchling.

Isolated blastomeres can divide and differentiate autonomously

These observations on PGC origin and development as traced with Vasa protein expression, combined with the cell lineage studies of other workers, indicate that "g" is specified as the exclusive germ line precursor as early as the eight-cell stage. At least two different molecular mechanisms could explain how "g" acquires this fate specification in early cleavage stages: (1) cytoplasmic germ line determinants may be present in the fertilized egg and asymmetrically partitioned to "g" during the first three cleavages, (2) "g," and/or the first and/or second cleavage blastomeres that will give rise to "g," may receive inductive signals from neighboring blastomeres before or at the eight-cell stage, which stimulate "g" to adopt germ cell fate. To distinguish between these two possibilities, I physically separated the first, second, and third cleavage blastomeres from each other, cultured them in isolation, and assayed their progeny for germ cell fate using Vasa protein as a marker.

There are two possibilities for the relative positioning of the first and second cleavage planes, and therefore two possible configurations of the second and third cleavage

Fig. 3. PGC proliferation during *P. hawaiensis* embryogenesis. A–P: confocal images of embryos stained with immunofluorescence: blue: nuclei; red: Vasa protein; green: anti-phospho-histone 3. (A) An early germ disc with approximately 175 cells has four PGCs at the anterior edge of the germ disc. (B) A 3D reconstruction of the embryo shown in panel A in oblique dorsal view, showing that PGCs are slightly dorsal to the main sheet of the germ disc (i.e., between the germ disc and the yolk). (C) A germ disc with approximately 300 cells and 15 PGCs clustered together close to the center. (D) A 3D reconstruction of the embryo shown in panel C in oblique dorsal view, showing that the PGCs are dorsal to the main sheet of the germ disc, in the bottom of the "bowl" formed by the germ disc curvature over the yolk. (E) High magnification of three PGCs at the germ disc stage, showing Vasa distribution in a punctate perinuclear pattern. (E′) Optical section through one of the PGC nuclei shown in panel E, demonstrating that Vasa protein is perinuclear and not inside the nucleus. (F) A germ disc with approximately 400 cells just beginning to elongate posteriorly. The PGC cluster has begun to elongate perpendicular to the anteroposterior axis. (G) High magnification 3D reconstruction of the ventral side of the embryo shown in (F), showing that PGCs are flanked anteriorly by multilayered ectodermal and mesendodermal cells, and posteriorly by a single layer of ectodermal cells. (H) An early germ band stage embryo with six to eight PGCs and scattered mitosis in most regions of the soma. (I) A later stage germ band embryo with 15 PGCs clustered between the head and trunk regions. (J) A 3D reconstruction of the embryo shown in panel I in dorsal view, showing that all PGCs remain underneath the ventral ectoderm. (J′ and J″) Orthogonal sections through the embryo shown in panel I. (K) 3D reconstruction of the embryo shown in panel I in full ventral view, showing the relative position of the PGCs (red) and the mesoblast clusters (false orange). (L) A late stage germ band embryo in which the PGCs have begun to split into two clusters to the left and right of the ventral midline. (M) Frontal view of an embryo at early limb bud development, in which some PGCs have already migrated to lateral positions (arrowheads) and others are still migrating away from the ventral midline (arrows). (M′) High magnification of the PGC cluster indicated with the left arrowhead in (M). (N) Frontal view of an "S" shape stage embryo in which all PGCs are found in one of two lateral clusters (arrowheads). (O) Lateral view of an embryo at the "comma" stage; one PGC cluster is seen underneath the lateral ectoderm (arrowhead). (P) Optical section through the PGC cluster of an embryo at the same stage as that shown in (O). (P′) High magnification of the PGC cluster shown in panel P. (Q–S) Whole mounts of immunohistochemically developed embryos. (Q) Dorsal view of an embryo at the late comma stage: PGCs are in two rounded dorsolateral clusters. Staining appears darker at the base of the appendages because of tissue thickness but is not specific: compare central and upper regions of the tissue shown in panel Q′. (Q′) Higher magnification of the area boxed in panel E shows PGCs closely associated with each other. (R) High magnification dorsal view of an embryo 1 day before hatching: PGCs are in two rounded clusters flanking the dorsal midline. (S) Dorsal view of an embryo at hatching: PGCs have elongated into a row (arrowheads). Dark spots in the anterior of the embryo are pigmented photoreceptor cells. Anterior is to the left in panels A–D, O–S, and up in F–N. Scale bars: A, C, D, F, H, I = 100 μm; L, M, N–P 50 = μm; E, M′, P′ = 10 μm; E′ = 2 μm; units in B, G, J, K = 50 μm. Number and thickness of confocal sections for 2D projections: A 49 × 2 μm; C 108 × 1 μm; E 10 × 2.4 μm; F 51 × 1 μm; H 100 × 1 μm; I 123 × 1 μm; L 64 × 2 μm; M 39 × 2 μm; N 58 × 1 μm; O 33 × 1 μm. Thickness of optical sections: E′ = 470 nm; M′ = 2 μm; P = 480 nm.

blastomeres, in mirror image to one another, illustrated in Fig. 4A. The first cleavage gives rise to two cells of slightly different sizes. The second cleavage is also asymmetric and can result in "Mvg," the smallest blastomere at the four-cell stage, being the sister cell of either "Eml" or "Emr." This means that at the two-cell stage, although the smaller of the two cells will produce "Mvg," it is not possible to tell whether its second daughter cell will be "Emr" or "Eml." The smaller first cleavage blastomere is therefore referred to as "MgRL," and the larger first cleavage blastomere as "EpenRL."

Blastomeres were freed from the surrounding egg membrane and separated from each other as shown in Fig. 4B. At the eight-cell stage, the blastomeres are unambiguously distinguishable from each other in intact embryos in almost all cases. However, in less than 5% of embryos, the size and morphology differences between micromeres are not readily apparent, leading to occasional misidentification (see below). At the four-cell stage, the differences between the blastomeres is somewhat more difficult to detect, and at the two-cell stage the margin of error rises slightly yet again. For this reason, sufficient dissections were performed so as

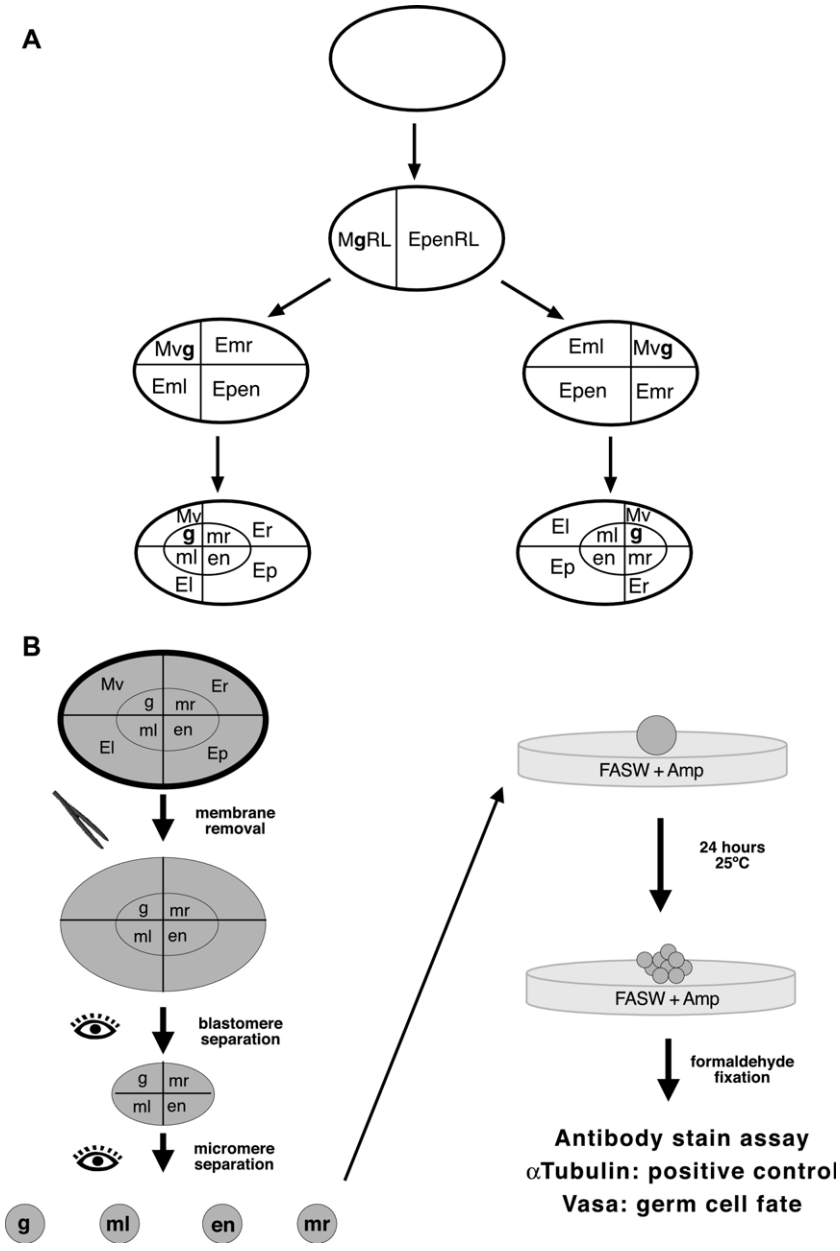


Fig. 4. Experimental determination of the mechanism of PGC specification. (A) Schematic diagram of the first three embryonic cleavages, showing the two possible mirror image embryos that occur with equal frequencies after second cleavage. (B) Experimental procedure and assay for developmental potential of isolated blastomeres with respect to germ cell fate. Forceps symbolize membrane removal with forceps; the eye symbolizes separation of blastomeres with an eyelash.

to give at least 20 scorable cases for each cell or group of cells studied (Fig. 5A).

After culturing for a minimum of 24 h at 25°C, the resulting aggregate of cells was fixed, and cellular integrity and germ cell fate were assayed by DIC imaging and immunohistochemistry. Twenty-four hours in culture at 25°C was chosen as the minimum time allowed for development of isolated blastomeres since an intact embryo raised under the same conditions would have reached an early germ disc stage (Fig. 1B), at which point two to eight PGCs should be clearly identifiable by staining with the Vasa antibody. Immunofluorescent staining with anti-α-tubulin was chosen as a positive control for normal cellular growth and morphology, as the cells of the early germ disc of intact embryos have a characteristic cortical distribution of α-tubulin, which clearly distinguishes them from the yolk mass and from each other (C. Extavour, unpublished observations).

The efficiency of the assay (fraction of isolated blastomeres surviving culture and processing for antibody staining) is shown in Fig. 5A. All control embryos were taken from the same broods as the isolated blastomeres and raised under the same conditions as the isolated cells. The morphology, mitotic division, and cell behavior of the progeny of isolated cells closely matched those of the equivalent cells in intact embryos at corresponding stages of development (Fig. 6). When the four micromeres of the eight-cell stage were separated from the macromeres but left in contact with each other, it was still possible to distinguish them from each other on the basis of their size and characteristic morphology (Fig. 6B). Individual third cleavage micromeres (Figs. 6C and D) and second cleavage blastomeres (Fig. 6E) tended to become ovoid or round a few minutes after being separated from the rest of the embryo, suggesting that cell shape is at least in part imposed by the embryonic membrane and the presence of neighboring cells. Blastomeres of the two-cell stage were isolated by ablating one of the cells and squeezing the cytoplasm of the ablated cell out of the embryonic membrane (Fig. 6F). Isolated blastomeres of this stage were generally left within a piece of the embryonic membrane, as they tended to stretch and rupture upon removal of the entire membrane. However, isolated first cleavage blastomeres, which were entirely freed of their membranes, developed identically to those whose membrane had been left on (Figs. 6L, R, and X).

One to two hours after blastomere isolation, control embryos had undergone a single cell division (Fig. 6G). Isolates observed at this time had also undergone a single cell division (Figs. 6H–L), indicating that the mitotic program of isolated blastomeres was maintained even in the absence of neighboring cells. The first division of isolated "Mvg" blastomeres from the four-cell stage was asymmetrical (Fig. 6K), as were the first and second divisions of isolated "MgRL" blastomeres from the two-cell stage (Figs. 6L and R), further demonstrating a cell autonomous mitotic program in early cleavage blastomeres.

At the stage when control embryo cleavage began to change from holoblastic to superficial (Fig. 6M), nuclei of isolate aggregates began to migrate towards the surface of the cells, accompanied by the surrounding cytoplasm (Figs. 6N–Q). By 24 h when the embryonic rudiment was forming in control embryos (Fig. 6S), all cells of isolate aggregates had risen to the surface of the yolk and were either scattered over the yolk globules or gathered together in one region of the aggregate (Figs. 6T–X). Cell movements were most striking in the progeny of isolated "MgRL" and "EpenRL" blastomeres (Fig. 6X), where by 24 h most cells of the

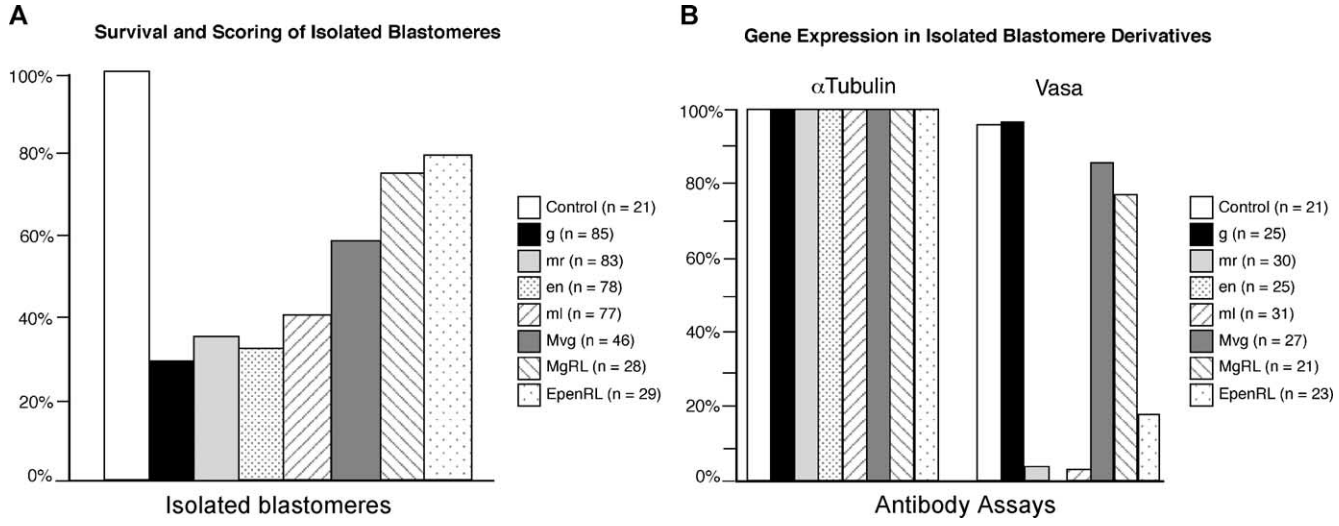


Fig. 5. Summary of blastomere isolation results. (A) Survival and scoring of isolated blastomeres, shown as the fraction of dissected and isolated blastomeres that survived culture and were scorable after antibody staining. Legend indicates total number of blastomeres isolated for each experiment. (B) Antibody assay of isolated blastomere derivatives, expressed as percentage of derivatives scored with positive staining. Blastomere progeny were assayed for normal cytoskeletal morphology with anti-α-tubulin antibody (left bars) and for germ cell fate with anti-Vasa antibody (right bars). Legend indicates total number of isolates scored.

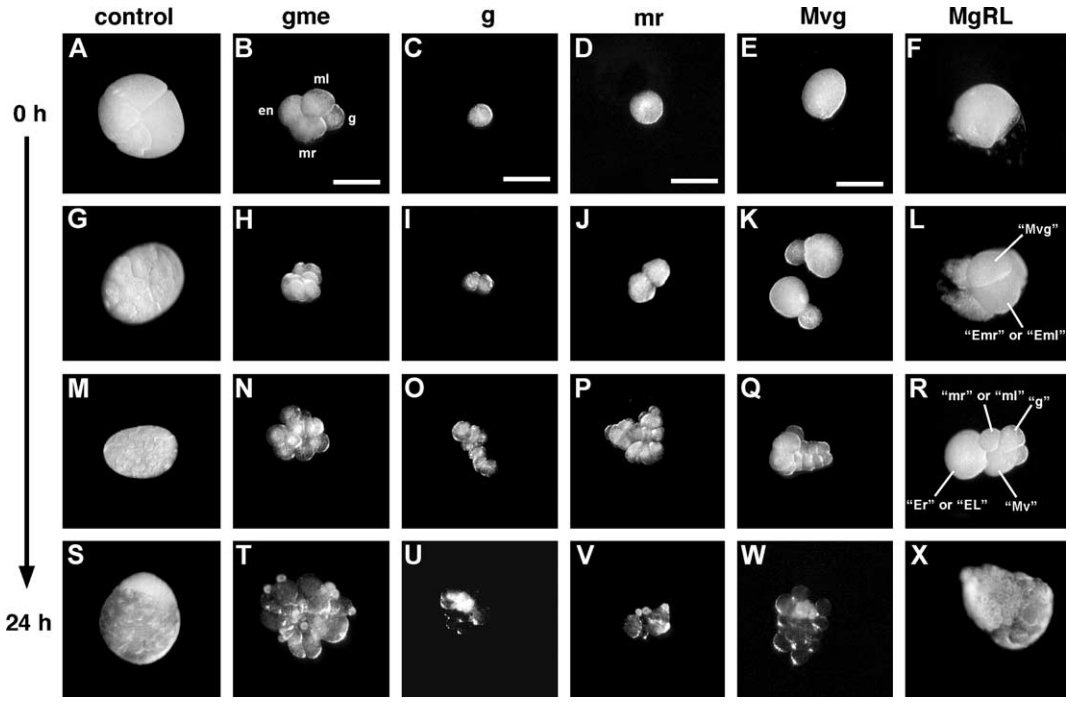


Fig. 6. Proliferation and cell behavior in isolated blastomeres. Blastomere identities are shown across the top, and time in culture progresses from the top to bottom rows. The first row shows control embryos (A) and isolated blastomeres (B–F) at the time of isolation. (G–L) Control and isolated cells following first cleavage in isolation. (M–Q) Control and isolates following several cleavages in isolation. (R) The result of asymmetric "third cleavage" in cells isolated at the two-cell stage. (S–T) The isolate progeny at the time of fixation for antibody assay. Scale bars: B–E 20 μm (applies to H–K, N–Q, and T–W).

aggregate had moved together to form a tiny "germ disc," and a few cells remained scattered over the remaining yolk in a manner reminiscent of the distribution of the extraembryonic cells in wild-type embryos. The cell lineage of *P. hawaiensis* indicates that each of the two cells at the two-cell stage should give rise to at least half of the ectoderm and half of the mesoderm (Gerberding et al., 2002), which may be capable of arranging themselves into germ layers without the progeny of the other half of the two-cell stage embryo. The behavior of the progeny of these cells in isolation suggests that the transition from holoblastic to superficial cleavage, and possibly even the gastrulation movements of these lineages, may also be cell autonomous properties.

"*g*" *gives rise to PGCs in the absence of contact with other blastomeres*

At the eight-cell stage, the four micromeres together (collectively called "gme") were separated from the macromeres and allowed to proliferate while retaining their cell–cell contacts during 24 h. In all cases ($n = 6$), the resulting aggregate of cells tended to cluster together on the surface of the excluded yolk globules (Fig. 7B), displayed normal α-tubulin distribution (Fig. 7H), and included two to four cells with perinuclear punctate Vasa staining (Figs. 7N and T). This suggests that at least some of the descendants of "g" are capable of producing germ cells in the absence of contact with the third cleavage macromeres.

To determine if "g" in the absence of the other third cleavage micromeres could also produce germ cells, aggregates of cells produced by "g" alone in culture were assayed for Vasa expression. Out of 85 "g" micromeres dissected and cultured, 25 aggregates were recovered and scored for germ cell fate. All but one aggregate of cells were positive for Vasa protein, displaying the characteristic perinuclear punctate distribution in between five and 14 cells (Figs. 5B and 7O, U). The observation that different "g" isolates cultured for the same amount of time gave rise to different numbers of germ cells is consistent with data from intact embryos, which at a comparable stage of development have produced anywhere from two to eight germ cells (data not shown). The single aggregate that did not stain positively for Vasa was most likely produced by one of the other three micromeres, misidentified during dissection as "g" (see also descriptions of "mr" and "ml" cultures below). Cellular and cytoskeletal morphologies were normal in all cells and the "g" descendants tended to cluster together in one region on top of the extruded yolk globules (Figs. 7C and I). With no exceptions, all of the progeny of "g" were Vasa positive. Eight-cell stage embryos in which only "g" was ablated developed germ discs that were wild type in cell number, morphology, and germ layer organization but which always lacked germ cells when assayed for Vasa protein ($n = 13$; data not shown). Taken together, these results indicate that (1) "g" produces germ cells in the absence of signals from any other third cleavage

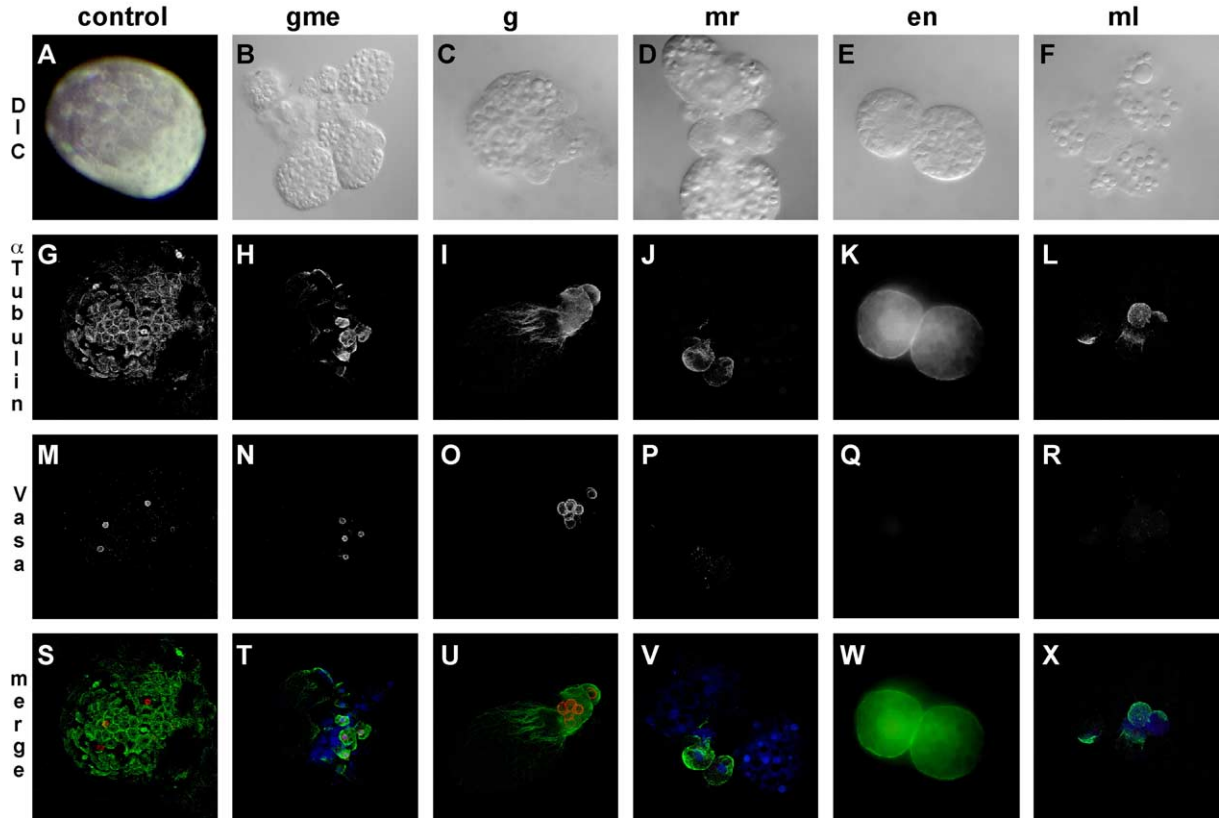


Fig. 7. Developmental potential of isolated third cleavage micromeres. Live morphology and staining of control embryo (germ disc stage) are shown in panels A, G, M, and S. All remaining panels show fixed, stained progeny of isolated micromeres. Only "gme" (N and O) and "g" (T and U) give rise to aggregates containing germ cells. In panels S–X, blue: nuclear stain (some yolk background in panels T, V, and X); red: Vasa protein; green: anti-α-tubulin.

blastomere, and (2) 100% of the progeny of "g" adopt germ cell fate.

It is a formal possibility that although in wild-type embryos the non-"g" micromeres contribute only to somatic tissues, they could produce germ cells when removed from the putative inhibitory influence of "g" or of the third cleavage macromeres. If this were the case, the germ cells observed in the aggregate derived from the four micromeres cultured together could have been partially or wholly the descendants of a micromere(s) other than "g." To test the capabilities of "mr," "en," and "ml" micromeres to produce germ cells when isolated from other third cleavage blastomeres, they were also cultured individually and their progeny assayed for germ cell fate.

As observed for the descendants of "g," the progeny of these isolated micromeres had normal morphological and cytoskeletal features and tended to cluster together on top of the extruded yolk (Figs. 7D–F and J–L). Their descendants were overwhelmingly Vasa negative. For the progeny of "en," which would normally produce endoderm, none of the cells was Vasa positive (n = 25) (Figs. 5B and 7Q, W). Progeny isolated from "mr" and "ml" micromeres were Vasa negative in almost all cases (n = 30 and 31, respectively) (Figs. 5B and 7P,R,V,X). The single exceptions to this rule were one "mr" and one "ml" aggregate, likely derived from misidentified "g" micromeres.

A germ cell determinant is localized to the first cleavage blastomere that will give rise to "g"

The experiments described above indicate that the third cleavage "g" micromere contains cell autonomous information for producing germ cells. To distinguish between (1) inheritance of this information through asymmetric localization of determinants in the second and/or first cleavage and (2) acquisition of germ cell fate through inductive signaling between blastomeres before third cleavage, I separated the blastomere that would normally give rise to "g" from its neighbor(s) at the four- and two-cell stages and assayed its progeny for germ cell fate.

At the four-cell stage, "Mvg" separated from the other blastomeres and cultured in isolation produced an aggregate of 15–25 cells, which clustered either in discrete clumps or all together, on the surface of the extruded yolk (Fig. 8A). The morphology and tubulin cytoskeleton of all cells was normal (Fig. 8D). Four to eight of the aggregate cells were positive for Vasa protein in 23 out of 27 cases (Figs. 5B and 8G,J). Since approximately 15% of four-cell stage embryos have blastomeres that are very difficult to distinguish from each other, I believe that the four Vasa-negative aggregates were in fact one of the other three blastomeres misidentified as "Mvg." Most of the cells of the aggregate were not Vasa positive, consistent with the cell lineage data indicating that

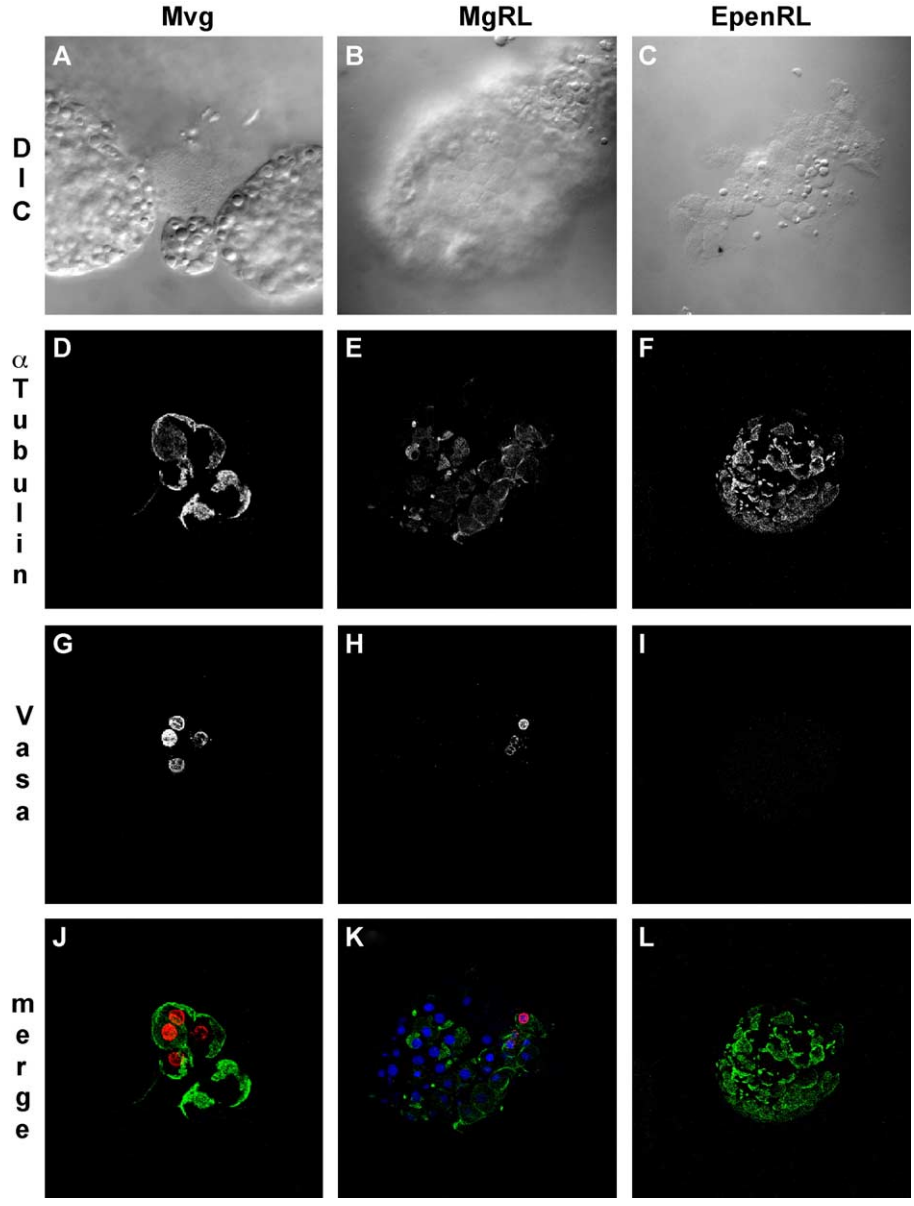


Fig. 8. Developmental potential of isolated first and second cleavage blastomeres. All panels show fixed, stained progeny of isolated cells. Only "Mvg" of the four-cell stage (G and J) and "MgRL" of the two-cell stage (H and K) give rise to aggregates containing germ cells. In panels J–L, blue: nuclear stain; red: Vasa protein; green: anti-α-tubulin.

the normal fate of third cleavage macromere "Mv" is to produce the visceral mesoderm (Gerberding et al., 2002). These data imply that the second cleavage "Mvg" macromere already contains a germ cell determinant, which is asymmetrically segregated to "g" at third cleavage. Taken together with the results of "g" ablation, I further conclude that when "Mvg" first divides asymmetrically (Fig. 6K), only the progeny of the smaller of its two daughter cells (the equivalent of the third cleavage "g" micromere) becomes germ cells.

To determine whether "Mvg" is induced to produce the germ cell determinant by contact with other blastomeres at or before the four-cell stage, or whether it inherits this determinant from its mother cell at the two-cell stage, I cultured isolated cells of the two-cell stage. Both "MgRL" and "EpenRL" (Fig. 4A) were cultured separately to determine the potentials of each first cleavage cell with respect to germ cell development.

"MgRL" grown in culture produced a small aggregate of approximately 45–65 cells, slightly less than half of the number of cells that would have been produced by an intact embryo at the corresponding stage of development. By 24 h at 25°C, the aggregate resembled a miniature version of the germ disc of an intact embryo grown under identical conditions (Fig. 8B). Most of the cells were gathered together into a roughly circular patch, while the remaining cells were scattered over the ball of excluded yolk. The miniature "germ disc" had most of its cells

arranged in a single layer, with a small fraction of the cells lying between the "disc" and the yolk. This is similar to the morphology of the early germ disc, in which a small founder population of endomesodermal and germ cells form an inner layer dorsal to the main ectodermal epithelium of the germ disc (Figs. 3A and B; Supplemental Data Movie 1).

The cytoskeletal morphology of the aggregate cells was normal (Fig. 8E), and in the majority of cases two to four of the cells were Vasa positive (Fig. 5B and 8H,K), comparable to the number of germ cells that were observed in control embryos. These data, combined with the results of isolations of the second cleavage "Mvg" and third cleavage "g" blastomeres described above, indicate that a germ line determinant is localized to the smaller of the two cells by shortly after first cleavage and asymmetrically segregated during subsequent cleavages to the "g" micromere at the eight-cell stage.

When "EpenRL" of the two-cell stage was cultured in isolation, its progeny formed a miniature "germ disc" similar in morphology and cytoskeletal architecture to that described above for "MgRL" (Figs. 8C and F). However, none of its progeny cells was Vasa positive (Figs. 8I and L), suggesting that the two blastomeres formed at first cleavage are not equal in potential with respect to germ cell formation, even when separated from each other shortly after cleavage.

Discussion

I have used Vasa protein expression as a marker to follow primordial germ cell specification, proliferation, migration, and gonad colonization throughout all embryonic stages of the amphipod crustacean *P. hawaiensis*. I have shown that all progeny cells of the smallest micromere of the eight-cell stage, "g," express Vasa protein, confirming the cell lineage data obtained by previous researchers suggesting that "g" was the exclusive germ line precursor. By isolating and culturing individual blastomeres at the two, four, and eight-cell stages, I have determined that germ cell fate is determined by asymmetric segregation of a germ line determinant during the first three cleavages to the "g" micromere of the eight-cell stage. These data show that only the "g" third cleavage micromere, or cells of the embryonic region that will give rise to "g" at third cleavage, produce cells that later adopt germ cell fate, as assayed by Vasa protein expression. Germ cell fate is autonomous to "g," which does not require contact with other cells to produce germ cells. Asymmetric localization of a germ line determinant occurs autonomously in the first and second cleavage blastomeres that will produce "g." Finally, other first and third cleavage blastomeres appear incapable of producing germ cells, either in intact embryos or when raised in isolation.

Preformation in germ cell development and localization of cytoplasmic determinants

What is the relationship between first cleavage and the segregation of the germ line determinant in *P. hawaiensis*? Cell lineage (Gerberding et al., 2002) and transgenesis studies (M. Averof and A. Pavlopoulos, personal communication) have demonstrated that the first cleavage not only segregates germ line fate but also corresponds to the axis of bilateral symmetry. The first cleavage is slightly off center with respect to the long axis of the egg. It is not clear whether the position of the first cleavage plane is random or fixed relative to a previously established marker of polarity. At least two scenarios are possible: (1) the egg is polarized at or before fertilization, and the first cleavage plane simply separates cytoplasmic determinants that are already localized; (2) the egg is not polarized, and embryonic asymmetry is achieved by interaction between the two first cleavage blastomeres. The site of polar body formation and the point of sperm entry have proved useful markers in studies of early embryonic polarity in many invertebrates (Goldstein and Freeman, 1997; Reverberi, 1971). Unfortunately, the dynamics of amphipod polar body formation and fertilization with respect to the first cleavage plane in *P. hawaiensis* do not suggest an obvious mechanism for establishment of anteroposterior polarity (Browne, 2003). Future studies of meiosis and fertilization will help to determine the timing of localization of germline and other putative cell fate determinants.

The nature of the germ cell determinant(s) in *P. hawaiensis*, and the molecular mechanism(s) of its localization, are as yet unknown. Amphipod ovaries do not have nurse cells that could supply the developing oocytes with maternal factors (Charniaux-Cotton, 1978; Schmitz, 1992; Zerbib, 1980), and there is no cytological or ultrastructural evidence for asymmetric localization of cytoplasmic factors, including Vasa protein, during gametogenesis (C. Extavour, unpublished). Studies on the mechanisms of cytoplasmic determinant localization have shown that the actin and tubulin components of the cytoskeleton, cytoplasmic flows, localized protein and mRNA degradation, cortical anchoring, and localized posttranslational modification can all contribute to polarization and germ cell specification in the zygote (Lyczak et al., 2002; Palacios and St. Johnston, 2001; Selwood, 2001; Wodarz, 2002). Investigations of early *C. elegans* embryos have shown that key aspects of cytoplasmic organization can be defined within a time window of 5–10 min during the one-cell stage (Hill and Strome, 1990). In *P. hawaiensis* embryos raised at 25°C, the time between fertilization and first cleavage is approximately 4 h, which is plausibly more than enough time for significant cytoplasmic rearrangement ensuring correct development of the germ line and other somatic lineages. Further studies of conserved germ cell components that may be present during the first few hours of development will provide

clues as to the mechanism of determinant localization in this amphipod.

Cell fate specification and developmental potential in crustaceans

How do *P. hawaiensis* early cleavage blastomere determination and germ cell fate commitment compare with these events in other crustaceans? Unfortunately, in contrast to the rich history of experimental embryology of non-arthropod marine invertebrates (Reverberi, 1971), relatively few studies have used physical manipulation of crustacean embryos to infer mechanisms of development. Centrifugation studies of beetle embryos showing that ectopic germ cells could be produced by shifting germ plasm to different regions of the embryo (Hegner, 1909), inspired similar experiments on embryos of the copepod *Cyclops viridis* (Stich, 1950). The latter studies essentially resulted in the embryonic rudiment being squashed into a small region of the egg but did not yield information about regional cell fate determinants. Ablation experiments at the eight-cell stage in amphipods suggested that macromere derivatives form most of the germ disc (Rappaport, 1960), which has been confirmed by subsequent cell lineage studies (Gerberding et al., 2002; Wolff and Scholtz, 2002). More recently, blastomere separations of two to 16-cell stage embryos of the shrimp *Sicyonia ingentis* showed that mesendoderm cells are autonomously specified by the four-cell stage (Hertzler et al., 1994). The germ line in this shrimp has been shown to derive from the mesendoderm but is probably not specified as early as the germ line in *P. hawaiensis*, as the *S. ingentis* germ line precursor arises from an asymmetric cell division of the ventral mesendoblast several cleavage cycles after the four-cell stage (Hertzler, 2002).

To my knowledge, the only non-malacostracan crustacean in which developmental potential of early blastomeres has been examined is the barnacle *Balanus*. When the blastomeres of the two-cell stage are separated from each other and cultured in isolation, both halves could give rise to a hatchling that was apparently complete, but half the size of animals hatched from intact embryos (Kajishima, 1951). This is a very different result to the one I obtained in *P. hawaiensis*, in which single blastomeres of the two-cell stage seem to be committed to produce distinct cell fates and do not show a regulative capacity to produce all embryonic cell types. However, without specific molecular markers for early somatic cell fate, the possibility that early cleavage blastomeres may regulate to replace missing somatic cell types cannot be ruled out. Experiments in which isolated blastomeres are reared long enough to analyze terminal differentiation phenotypes (e.g., muscle, cuticle) are currently technically challenging (C. Extavour, unpublished observations) but will be required to definitively test the regulative capacities of these cells. In further experiments, it will be necessary to monitor the timing of cell separation relative to first cleavage, to determine whether *P. hawaiensis* first cleavage blastomeres are truly autonomous with respect to germ cell differentiation. It will also be critical to gather experimental data on the early embryology of members of other crustacean classes, particularly the primitive Branchiopoda, to understand which developmental mechanisms might have been shared by a common crustacean ancestor.

Evolution of germ cell specification in arthropods

Because most well-studied model organisms specify their germ cells early in embryonic development using localized cytoplasmic determinants (preformation), it is generally assumed that preformation is the rule and the ancestral mode for all metazoans. However, wider surveys of the literature indicate that epigenetic induction of germ cell fate, as observed in mice, may be more common than is currently apparent from studies of genetic model organisms (Extavour and Akam, 2003; Nieuwkoop and Sutasurya, 1979, 1981).

The present study clearly shows that in *P. hawaiensis*, germ cell specification is achieved by asymmetric localization of determinants during early cleavages. These data, combined with data for flies, could be interpreted to suggest that at least for the Pancrustacea (Crustaceans + Hexapods), preformation of the germ line is an ancestral characteristic. However, embryonic development in amphipods shows many derived characteristics, even with respect to other malacostracan crustaceans (Anderson, 1973; Gerberding et al., 2002; Wolff and Scholtz, 2002). While the phylogenetic position of the Malacostraca within crustaceans is unclear, they are unlikely to be the most primitive of crustacean classes (Edgecombe et al., 2000; Schram, 1986). Amphipods might therefore be considered to be a poor representative for generalized crustacean development, just as *Drosophila* is a poor model for generalized insect development. On the other hand, recent data suggest that the Collembola may derive from a lineage basal to the last common ancestor of insects and crustaceans (Nardi et al., 2003) (C. Cook, personal communication). In collembolans, segregation of germ cells by preformation is supported by several detailed histological and ultrastructural studies (Claypole, 1898; Garaudy-Tamarelle, 1969, 1970; Klag, 1982; Klag and Swiatek, 1999).

For arthropods other than the Pancrustacea, there are only sparse data on germ cell specification, and it is difficult to conclude anything about ancestral mechanisms from the existing literature. For example, in some spiders, researchers have reported early segregation of germ cells (Montgomery, 1909), while most investigators of myriapod embryology were unable to detect PGCs until late segmentation stages of development (Heymons, 1901). Additional studies on more diverse representatives of all major arthropod groups, combined with further phylogenetic analysis, will be necessary to determine which mode of germ cell specification is ancestral to arthropods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.09.030.

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